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Life Sciences 72 (2003) 989–1000

Life Sciences

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Real-time measurement of nitric oxide by luminol-hydrogen peroxide reaction in crystalloid perfused rat heart

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Received 20 December 2001; accepted 2 July 2002

Abstract

The objective of this study was to develop an assay system that allows continuous monitoring of nitric oxide (NO) released from crystalloid perfused hearts. We utilized chemiluminescence reaction between NO and luminol-H_2O_2 to quantify the NO level in coronary effluent. Isolated rat hearts were subjected to ordinary Langendorff's perfusion, and the right ventricle was cannulated to sample coronary effluent. After equilibration, the coronary flow rate was set constant and the hearts were paced at 300 bpm. Coronary effluent was continuously sampled and mixed with the chemiluminescent probe containing 0.018 mmol/l luminol plus 10 mmol/l H_2O_2. Chemiluminescence from the mixture of coronary effluent and the probe was continuously measured. NO concentration was calibrated by various concentrations (0.5–400 pmol/l) of standard NO solution. The lower detection limit of NO was 1 pmol/l. Basal NO release from isolated perfused rat heart was 0.41 ± 0.17 pmol/min/g of heart weight, and that was significantly suppressed by 0.1 mmol/l of L-NAME to 0.18 ± 0.10 pmol/min/g of heart weight (n = 7). Application of 0.1 and 0.3 μmol/l acetylcholine increased NO level in the coronary effluent, in a concentration-dependent manner, from 6.6 ± 1.7 in a baseline condition to 16.3 ± 7.4 and 30.3 ± 16.1 pmol/l at each peak, respectively. Thrombin at 1 and 10 U/ml also increased NO level from 17.6 ± 4.3 in control to 35.5 ± 10.4 and 48.7 ± 8.7 pmol/l at each peak, respectively (n = 7). Thus, this assay system is applicable to the continuous real-time measurement of NO released from crystalloid perfused hearts, and it may be useful for the study of physiological or pathophysiological role of NO in coronary circulation.
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Keywords: Nitric oxide; Coronary circulation; Kinetic measurement; Luminol-H_2O_2 reaction; Perfused rat heart; Thrombin

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PII: S0024-3205(02)02353-6

Introduction

Nitric oxide (NO), originally described as the endothelium-derived relaxing factor [1,2], has been shown to involve in a number of physiological processes as well as in pathophysiological conditions in various tissues and cell types. In coronary circulation, NO is constitutively produced in the vascular endothelium from L-arginine and oxygen through calcium-dependent nitric oxide synthase (eNOS), and plays a major role in maintaining coronary vascular tone. Many factors have been shown to regulate the NO production; including neurohormonal factors such as acetylcholine, bradykinin, norepinephrine, thrombin, adenosine trisphosphate, serotonin and histamine; fatty acids; ionophores; and shear stress [3]. NO has been implicated also in the pathogenesis of several cardiovascular diseases: an impairment of NO production may play a role in hypertension or atherosclerosis, and conversely excess of NO may participate in ischemia reperfusion injury [4]. Therefore, it is important to quantify the NO production to understand the mechanism by which NO mediates various physiological and pathophysiological processes in the heart.

Since NO has a very short half-life (<6 sec) in vivo, the continuous real-time measurement of NO release is a challenging problem, especially under physiological conditions where NO production is relatively low [5,6]. Several detection systems have already been tried to this end; utilizing either electron paramagnetic resonance (EPR) spectroscopy [7,8], chemiluminescence reaction with ozone [9], oxyhemoglobin method [10], or NO electrode [11]. However, none of these methods has ever succeeded in providing adequate sensitivity and/or specificity for NO detection[12]. Kikuchi and his colleagues [13–16] have reported that the chemiluminescence reaction of NO with luminol-H_2O_2 is usable for the measurement of NO released from a perfused rat kidney. NO and H_2O_2 make peroxynitrite ($ONOO^-$), which then reacts with luminol, thereby generating chemiluminescence with peak emission at 302 nm (Fig. 1). They have demonstrated also that this method allows detecting NO as low as at 100 fmol/l concentration, and that the method is applicable to the continuous real-time measurement of NO release with adequate specificity [13,14]. Moreover, they have recently extended its application to cultured endothelial cell as well as living rat brain [16,17]. Employing this method, we have developed a detection system for continuous real-time measurement of NO released from a perfused rat heart, and investigated the changes of NO release in response to pharmacological agents such as acetylcholine and thrombin.

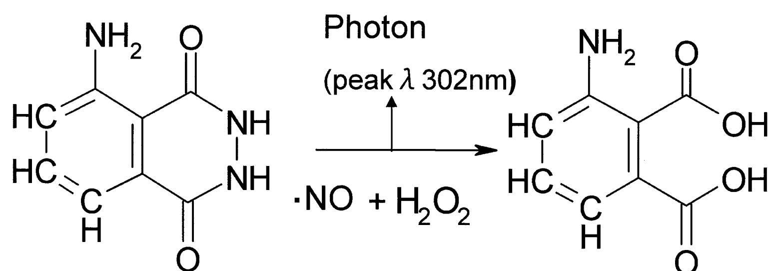


Fig. 1. Luminol chemiluminescence reaction with NO and H_2O_2.

Methods

Animals and reagents

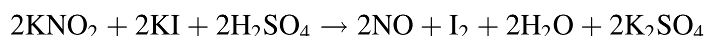
Male Wistar rats (250–300 g) were obtained from Saitama Experimental Animal Supply Co., Ltd. (Saitama, Japan). Luminol purified for chemiluminescence assay, H_2O_2 and N-ω-nitro-L-arginine-methyl ester (L-NAME) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan); desferrioxamine from Sigma (St. Louis, MO, USA); acetylcholine from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan); thrombin from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Animals were treated according to the Guidelines of Nippon Medical School for Regulation of Animal Experimentation (1988).

Chemiluminescent probe

The chemiluminescent probe for the measurement of NO was prepared as described in the literature [13]. In brief, luminol was dissolved in an alkaline solution with deionized water plus K_2CO_3, and diluted with ice-cooled water containing H_2O_2 and desferrioxamine. The chemiluminescent probe composition was as follows (mmol/l at final concentration): luminol 0.018, H_2O_2 10, desferrioxamine 0.15 and K_2CO_3 2. K_2CO_3 was used to raise pH because the luminol-H_2O_2 reaction exhibits stronger emission in an alkaline condition (emission peak at pH 12). Desferrioxamine was added to quench heme interference by hemoglobin with luminol-H_2O_2 chemiluminescence reaction.

Standard NO solution

The standard NO solution was prepared according to the method by Schmidt and Mayer [18], which is based on the following reaction of nitrite with iodide in sulfuric acid:



$$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2K_2SO_4$$

To produce 400 pmol/l NO solution, 7.2 ml of 0.1 mmol/l KNO_2 solution was added to 1.8 ml of helium gassed solution with 0.1 mol/l KI and 0.1 mol/l H_2SO_4 at 25 °C.

The dilution series was made from this NO solution, with NO concentrations ranged from 0.5 to 400 pmol/l. A standard calibration curve was constructed from this series.

Perfused heart preparation

A rat was anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and 200 units of sodium heparin was injected through the femoral vein. The heart was excised and immediately placed in a cold perfusion solution (4 °C) until contraction ceased. It was cannulated via the aorta and perfused by the Langendorff's method. The perfusion solution was a bicarbonate buffer of the following composition (mmol/l): NaCl 118.5, KCl 4.75, $NaHCO_3$ 25.0, $MgSO_4$ 1.20, KH_2PO_4 1.20, $CaCl_2$ 1.40 and glucose 11.1. All perfusion solutions were filtered (pore size 5 μm) and bubbled continuously with 95%O_2 + 5%CO_2 at 37 °C.

Measurement of NO, coronary perfusion pressure and flow rate

As depicted in Fig. 2, coronary effluent sampled at 2 ml/min by a peristaltic pump was mixed with the chemiluminescent probe using a micro-syringe infusion pump at an infusion rate of 10 μl/min (BS-MD 1001, Bioanalytical Systems, USA). The mixture was pumped into chemiluminescence detector system, employing a chamber-type photomultiplier-detector (Hamamatsu-Photonics, Shizuoka, Japan) and a photon counting system (model PHC-2001, Scientics Co., Shizuoka, Japan). Transit time from the right ventricle to photomultiplier-detector was ≈ 15 sec. The acquired chemiluminescence signal from the mixture was digitized after current-voltage conversion. Coronary perfusion pressure (CPP) was monitored through a side-arm of the aortic cannula, and total coronary flow rate was also monitored using an in-line flow detector (Transonic T206 Animal Research Flowmeter with 2N probe, Transonic Systems, Ithaca, NY, USA). These data were stored in a MO disk using MP-100 data acquisition system (Biopac Systems, Santa Barbara, CA, USA) and analyzed with the Acqknowledge® Software (version 3.5.3).

Experimental protocols and data analysis

Following the initiation of Langendorff perfusion, the heart was paced at 300 bpm via the right atrium, and a 23 G needle was cannulated from the right atrium into the right ventricle to sample coronary effluent. After 30 min of perfusion at 75 mmHg for equilibration, perfusion was changed to

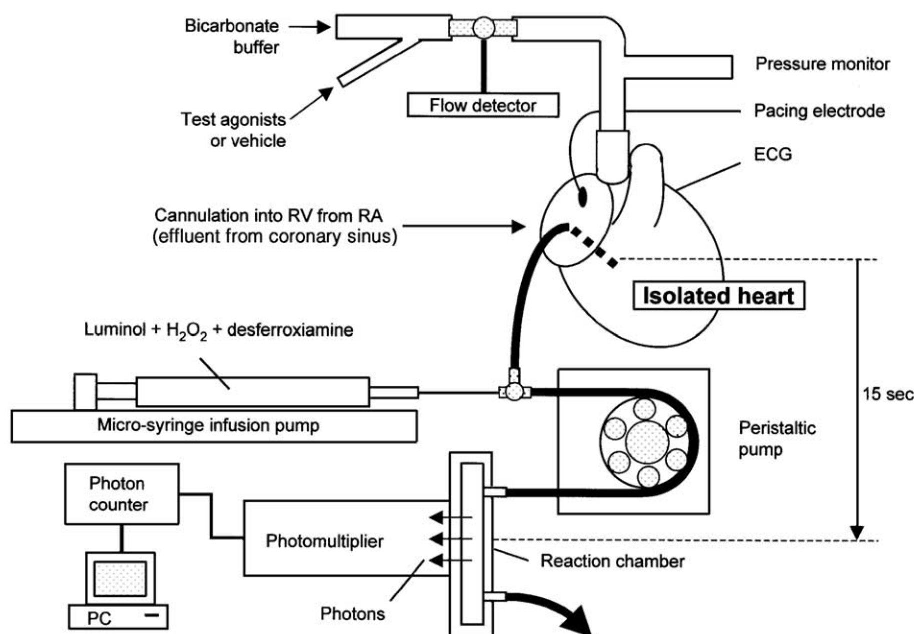


Fig. 2. Schematic presentation of the whole experimental set-up. Coronary effluent was continuously sampled from a crystalloid perfused rat heart and pumped into the assay system. To avoid any toxic effects, the chemiluminescent probe (luminol-H_2O_2) was mixed after the sample exited the heart. The lag time (15 sec) from the sampling point to the reaction chamber allows tissue-derived superoxide or peroxynitrite to decay to the negligible level. RV: right ventricle, RA: right atrium.

constant flow mode using a Gilson M312 pump (Gilson, France). Coronary flow rate was held constant at a value noted at the end of the equilibration for each heart (mean flow rate: 12.5 ± 1 ml/min). When stable chemiluminescence signal was obtained, the photon counts for 180 sec were averaged and converted to NO concentration. Basal NO release (pmol/min/g of heart weight (g HW)) was calculated by multiplying the value by coronary flow rate and then dividing its product by heart weight (determined at the end of experiment). In some hearts, 0.1 mmol/l L-NAME was included in the perfusion solution in order to investigate the role of eNOS on the basal NO release.

Ligand mediated stimulation of NO release was also examined using acetylcholine and thrombin. When required, either acetylcholine or thrombin was supplied from a side-arm (7% of the total flow) at a low and a high concentration for 2 min each with more than 5-min interval. The concentrations used were, 0.1 and 0.3 μmol/l for acetylcholine, 1 and 10 U/ml for thrombin. In some hearts, effect of thrombin on NO release was examined also in the presence of 0.1 mmol/l L-NAME (L-NAME infusion was started at least 30 min before the first challenge of thrombin). All agents were dissolved directly in the perfusion solution.

As indices of NO production in response to those agonists, the peak NO concentration of the coronary effluent (pmol/l) and the net increase in NO release (ΔNO increase) were assessed. The ΔNO increase was defined as a mean net increase in NO concentration of the coronary effluent in response to each agonist, multiplied by coronary flow rate and divided by each heart weight (expressed as pmol/min/g HW).

Statistical analysis

All values are presented as means ± SEM. Data were analyzed by paired Student's *t*-test. When required, Bonferroni's correction was performed. A probability of less than 0.05 was considered statistically significant.

Results

Standard curve for NO measurement

The emission intensity of the standard NO solution increased when its mixing process with the chemiluminescent probe started. As shown in Fig. 3, signal intensity was linearly correlated to the NO concentration between 1 and 400 pmol/l (R = 0.995). The lower detection limit was ≈ 1 pmol/l in this system because the signal increase by 0.5 pmol/l of standard NO solution was too small and unable to fit on the same line.

Measurement of NO concentration and perfusion pressure from isolated perfused heart

Coronary perfusion pressure and chemiluminescence intensity became steady after a 30 min equilibration period. Basal NO release from isolated perfused rat heart was 0.41 ± 0.17 pmol/min/g HW, and that was significantly suppressed by 0.1 mmol/l of L-NAME to 0.18 ± 0.10 pmol/min/g HW (n = 7) (Fig. 4).

As depicted in Fig. 5, a dose-dependent increase in NO release occurred by acetylcholine. However, CPP did not decrease but increase upon infusion of acetylcholine. The peak NO concentrations and

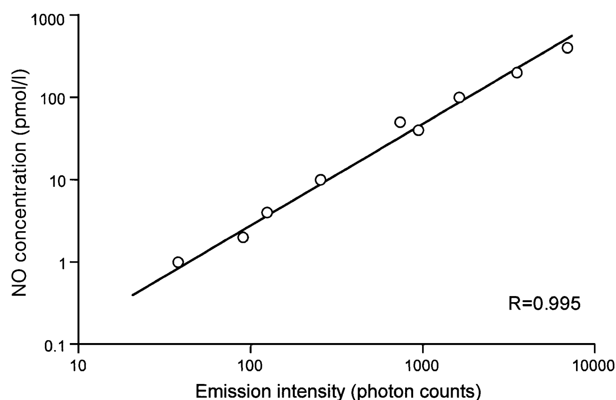


Fig. 3. Standard calibration curve for NO concentration (pmol/l).

the ΔNO increases after application of 0.1 and 0.3 μmol/l acetylcholine (n = 5) were 16.3 ± 7.4 and 30.3 ± 16.1 pmol/l (Fig. 6A), and 0.23 ± 0.04 and 0.38 ± 0.99 pmol/min/g HW (Fig. 6B), respectively.

Thrombin also increased NO release in a dose-dependent manner with concomitant changes in CPP. CPP exhibited a biphasic response: an initial rapid decrease just after the initiation of thrombin infusion followed by the gradual elevation exceeding the control level (Fig. 7). The peak NO concentrations after thrombin challenge at 1 and 10 U/ml (n = 7) were 35.5 ± 10.4 and 48.7 ± 8.7 pmol/l, respectively (Fig. 8A). Those values were significantly higher than the baseline NO level before the thrombin

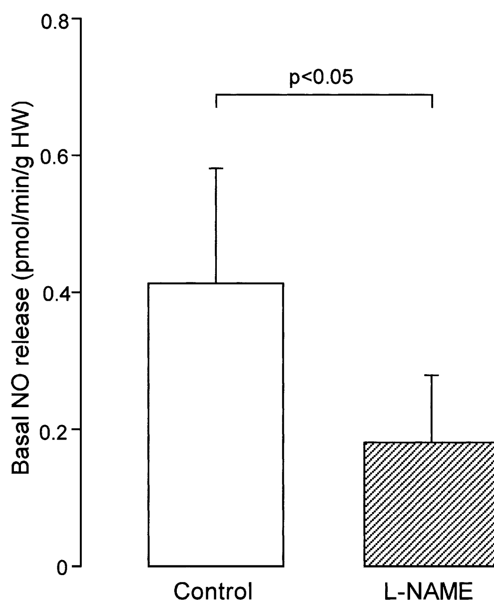


Fig. 4. Basal NO release from a perfused rat heart in the absence (open column) or presence (hatched column) of 0.1 mmol/l L-NAME. Data were expressed as mean ± SEM. HW: heart weight (n = 7).

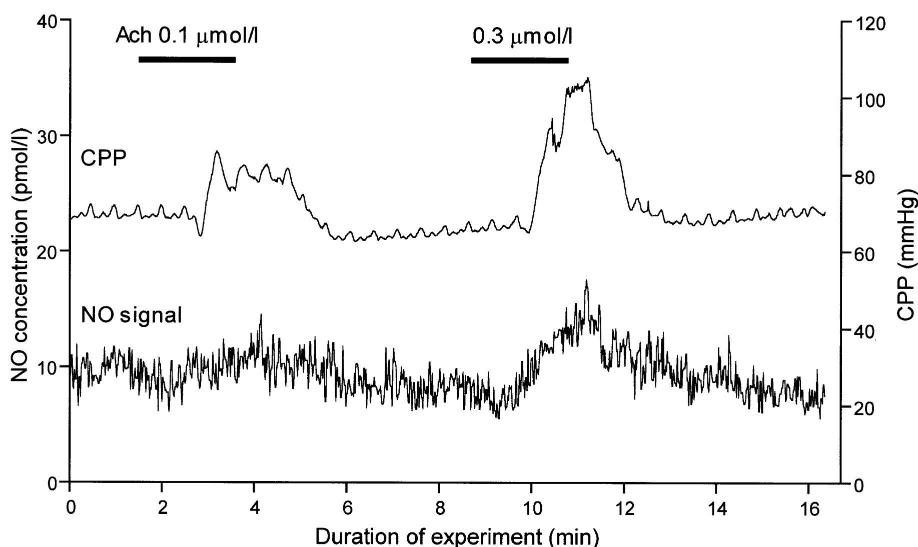


Fig. 5. Representative recording of temporal changes in NO concentration and coronary perfusion pressure (CPP) in response to acetylcholine (Ach).

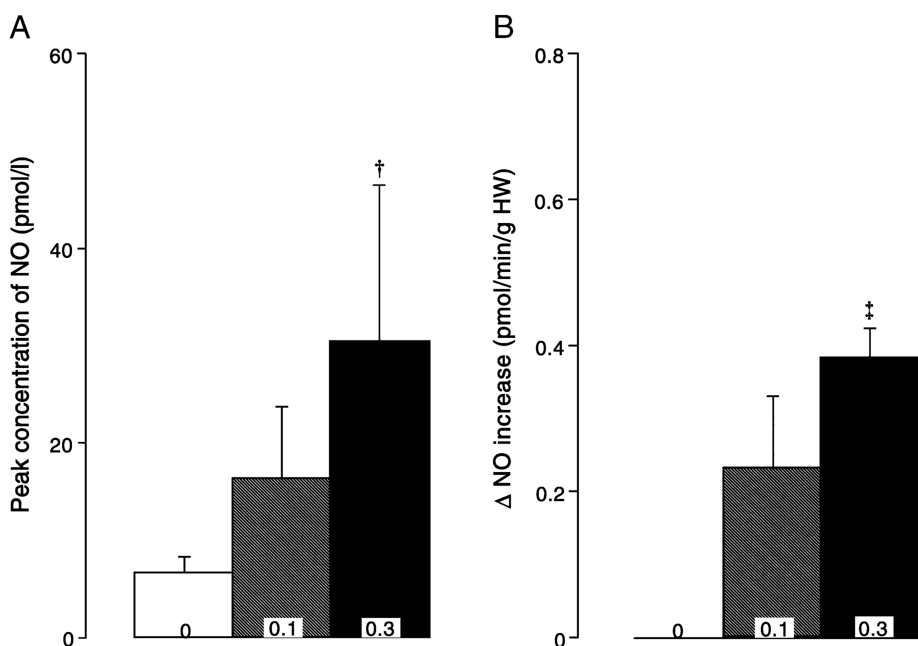


Fig. 6. Effects of acetylcholine on the peak concentration of NO (panel A) and the ΔNO increase (panel B). Acetylcholine was applied at 0.1 (hatched columns) and 0.3 μmol/l (black columns). White column indicates the baseline concentration just before the drug challenge. Data were expressed as mean ± SEM. HW: heart weight. (†$p < 0.1$ versus baseline value, ‡$p < 0.1$ versus 0.1 mmol/l acetylcholine, n = 5).

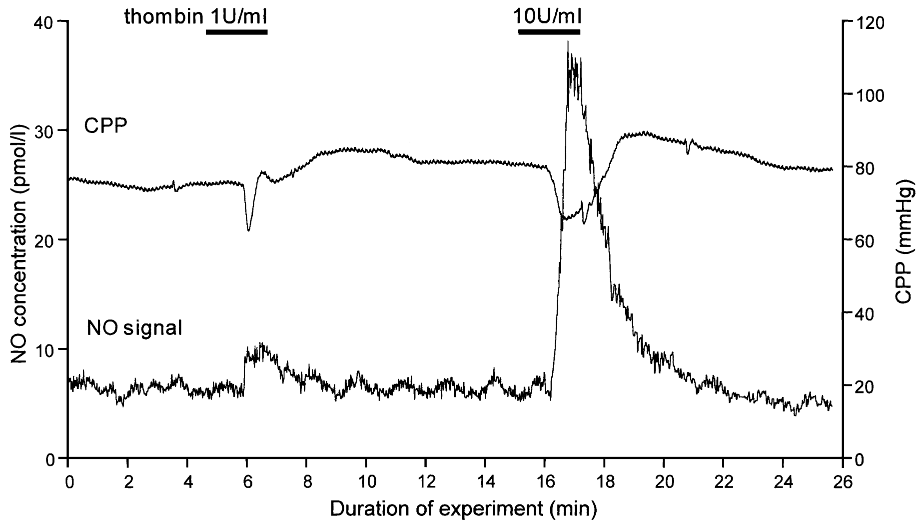


Fig. 7. Representative recording of temporal changes in NO concentration and coronary perfusion pressure (CPP) in response to thrombin.

administration (17.6 ± 4.3 pmol/l). In addition, treatment with 0.1 mmol/l L-NAME tended to suppress the peak NO concentration induced by thrombin, whereas stimulatory effect of thrombin on the NO release did not disappear even in the presence of L-NAME: the peak NO concentration were 22.0 ± 8.9 and 26.9 ± 4.8 pmol/l for 1 and 10 U/ml thrombin as opposed to 11.4 ± 5.6 in a baseline condition

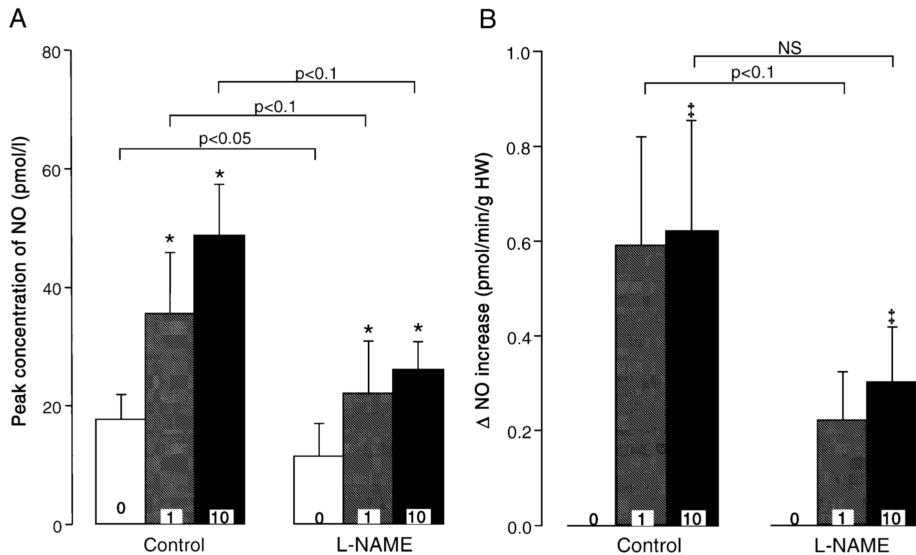


Fig. 8. Effects of thrombin with and without 0.1 mmol/l L-NAME on the peak concentration of NO (panel A) and the ΔNO increase (panel B). Thrombin was applied at 1 (hatched columns) and 10 U/ml (black columns). White column indicates the baseline concentration of NO just before the drug challenge. Data were expressed as mean ± SEM. HW: heart weight. (*p < 0.05 versus baseline value; ‡p < 0.1 versus 1 U/ml thrombin; n = 7).

(Fig. 8A). Similar tendency was observed also in the ΔNO increase: the values by thrombin at 1 and 10 U/ml were 0.59 ± 0.23 and 0.64 ± 0.23 in the absence of L-NAME, 0.22 ± 0.10 and 0.30 ± 0.12 pmol/min/g HW in the presence of L-NAME (Fig. 8B).

Discussion

We have demonstrated that the method, in which NO is determined by a chemiluminescence reaction of NO with luminol-H_2O_2, can be applied for continuous real-time measurement of NO released from isolated crystalloid perfused rat hearts. This approach enabled us to quantify NO released into the coronary vasculature and also to assess its release-kinetics under the conditions we studied. The present study also provided direct evidence that thrombin induces coronary vasorelaxant effect through the production of NO.

Measurement of NO by luminol and hydrogen peroxide chemiluminescence reaction

Kikuchi and colleagues [13–17] reported that the chemiluminescence reaction of NO with the luminol-H_2O_2 was applicable to the detection of NO in a variety of biological samples. As noted earlier, NO reacts with H_2O_2 to form peroxynitrite ($ONOO^-$). Peroxynitrite then reacts with luminol, thereby generating chemiluminescence with the peak emission at 302 nm. Using this reaction as an assay principle, they succeeded in continuously measuring NO release from isolated perfused rat kidneys [14,15]. The lower limit of detection was ≈ 100 fmol/l. Then, they concluded that their assay system was the most sensitive real-time NO assay technique in physiological buffer solutions of all available methods [14]. In our study, the standard calibration curve revealed that the signal intensity was linearly correlated to the NO concentration between 1 and 400 pmol/l, and that the detection limit was as low as 1 pmol/l. Although our system appeared to be 10-fold less sensitive than that in the previous report [14], the sensitivity was still greater than that of any other available methods for NO detection (see later discussion).

Since tissue-derived superoxide and peroxynitrite generate luminol chemiluminescence, one might argue that these radicals can interfere with the measurement of NO. However, this possibility has been discussed and theoretically excluded in the studies by Kikuchi et al [13,14]. First, they looked at the effect of Mn containing superoxide dismutase (Mn-SOD) on the NO level measured by luminol-H_2O_2 chemiluminescence reaction under various conditions. Co-infusion of Mn-SOD up to 250 U/ml did not show any significant effect on the NO level, whereby suggesting little role of superoxide in their model. Second, in O_2-saturated Krebs-Henseleit buffer, superoxide and peroxynitrite have at least 3-fold shorter half-lives than that of NO (< 1.9 vs. 6.4 sec). It is hence unlikely that both free radicals would be present at a detectable level after the15-sec lag time that it takes for sample to reach the reaction chamber from the heart. Therefore, it is reasonable to assume that the effects of these species on chemiluminescence reaction would be negligible.

Measurement of NO from isolated perfused heart

Several studies have reported the measurement of NO in perfused hearts, using either electron paramagnetic resonance (EPR) spectroscopy [7,8], chemiluminescence reaction with ozone [9], oxy-

hemoglobin method [10], or NO electrode [11]. EPR spectroscopy offers high specificity for NO detection, however, it is technically demanding and allows only micromolar order of detection. The oxyhemoglobin method has a lower detection limit of ≈ 1 nmol/l, but it is inferior in its specificity to the EPR spectroscopy because oxyhemoglobin reacts NO_2^- as well as NO. Chemiluminescence reaction with ozone provides similar sensitivity to oxyhemoglobin method, but it is also short of specificity for NO. NO selective porphyrinic microsensor is reported to be usable also for real-time measurement of NO, but its detection limit is ≈ 10 nmol/l [11]. In comparison with these methods, our system using luminol-H_2O_2 chemiluminescence reaction appear to be far more sensitive than any other method available for real-time measurement of NO.

In the present study, acetylcholine, which is a classical stimulator of NO release, enhanced NO production from the heart in a concentration-dependent manner. Despite such an increase of NO release, CPP paradoxically increased. This may well be explained by the opposing actions of acetylcholine on vascular smooth muscle cells. Acetylcholine has both direct vasoconstrictor action and indirect vasodilator action, and the net response results from the sum of these two actions [19]. In perfused rabbit or guinea-pig hearts, acetylcholine induces coronary vasodilatation [20–24]. In contrast, in perfused rat hearts, acetylcholine and other cholinergic stimulants induced vasoconstriction in the capillary beds of intact coronary arteries with an endothelial lining [25,26]. Thus, acetylcholine appears to have a complex effect on the coronary circulation, depending upon the species. It is therefore speculated that, under our study conditions, the direct vasoconstrictor effect by acetylcholine overwhelmed the indirect vasodilating action via the production of NO.

Thrombin-induced enhancement of NO release

Various agents other than acetylcholine are known to stimulate the release of NO [3]. Our data showed a thrombin-induced dose-dependent increase in NO release from perfused rat heart, which was suppressed by the pre-treatment with L-NAME. CPP after the infusion of thrombin exhibited a biphasic response: an initial dip followed by a reversible heave. To our knowledge, this is the first to demonstrate that thrombin enhances NO release from the coronary vessels with a biphasic change in coronary vascular tone. It has been shown that thrombin elicits versatile biological actions in a variety of cell types, including vascular smooth muscle cells, endothelial cells, and platelets [27–29]. Studies using rings of rat aorta have suggested that thrombin modulates vascular tone through production of both NO and endothelin-1 [30,31]. The biphasic response of CPP in response to thrombin observed in our study may be explained by the release of these vasoactive substances.

Limitation of the system

There are a couple of methodological limitations as described in the literature [13]. Because the NO release is determined by NO concentration of the sampled perfusate, flow rate through the tissue and the tissue weight, this system is recommended for experiments with constant flow (the continuous real-time measurement is difficult unless flow rate is fixed). Besides, the signal artifacts are reported to increase when flow rate varies. Another limitation of this method is that the assay cannot be performed in the presence of hemoglobin. Therefore, the system can be used only in isolated crystalloid perfused organs where blood has been thoroughly washed out.

Conclusion

The application of the luminol-H_2O_2 chemiluminescence method for real-time monitoring of NO is successfully extended to the crystalloid perfused rat heart preparation. Knowing its superiority and limitations, investigators may well go on to utilize this method for further understanding of physiological or pathophysiological roles of NO in coronary circulation.

Acknowledgements

The authors are grateful to Dr. Kazuya Kikuchi for valuable comments on our manuscript.

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